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Different Expression Patterns of Toll-Like Receptor mRNAs in Blood Mononuclear Cells of IgA Nephropathy and IgA Vasculitis with Nephritis

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Mucosal immunity may play a key role in IgA nephropathy (IgAN) and IgA vasculitis with nephritis (IgAVN). IgAVN is characterized by the presence of non-thrombocytopenic palpable purpura, associated with glomerulonephritis with IgA-dominant immune deposits. Recent studies have shown the up-regulation of Toll-like receptors (TLRs) in patients with IgAN or IgAVN. Among TLRs that mediate innate immune reactions, TLR2, TLR4, and TRL5 recognize bacterial components, while TLR3, TLR7, and TLR9 recognize viral components. Here we compared the expression levels of *TLR* mRNAs in peripheral blood mononuclear cells (PBMCs) from 49 IgAN patients, 20 IgAVN patients, and 20 patients with thin basement membrane nephropathy (TBMN), unrelated to immune-mediated pathogenesis, as a control. The real-time RT-PCR analysis revealed the significantly higher expression levels of *TLR2*, *TLR3*, *TLR5*, *TLR7*, and *TLR9* mRNAs in PBMCs of IgAN and IgAVN patients, compared to TBMN patients. Importantly, *TLR4* mRNA levels were significantly higher in IgAN patients than in IgAVN patients, while its expression levels were comparable in IgAVN patients and TBMN patients. In contrast, *TLR5* and *TLR9* mRNA levels were significantly higher in IgAVN patients than in IgAN patients. In IgAN patients, expression levels of *TLR2*, *TLR3*, *TLR5*, or *TLR9* mRNA were correlated with proteinuria levels, and *TLR4* mRNA levels were correlated with serum IgA levels. In IgAVN patients, however, there was no such correlation. The up-regulated expression of *TLR* mRNAs in PBMCs may be related to the development of IgAN and IgAVN. The distinct expression patterns between these two diseases may reflect their different pathogenetic mechanisms.

Keywords: IgA nephropathy; IgA vasculitis; peripheral blood mononuclear cells; real-time reverse transcription polymerase chain reaction; Toll-like receptors
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Introduction

IgA nephropathy (IgAN) is the most prevalent primary chronic glomerular disease worldwide. The spectrum of the clinical manifestation of IgAN is expansive, ranging from sustained asymptomatic microscopic hematuria to gross hematuria during an upper respiratory or gastrointestinal infection. It has been established that some clinical parameters are strong indicators of severe disease progression, such as hypertension, moderate to severe proteinuria, and increased serum creatinine concentration (Wyatt and Julian 2013; Zhang et al. 2015). IgAN is characterized histologically by expansion of the glomerular mesangial matrix with mesangial cell proliferation and/or mononuclear cell infiltration. The glomeruli contain predominant or co-dominant granular IgA deposits, usually with complement C3 and variable amounts of IgG and/or IgM.

On the other hand, IgA vasculitis (IgAV) (Jennette et al. 2013), previously known as Henoch-Schönlein purpura, is a systemic small vessel vasculitis. It is characterized by non-thrombocytopenic palpable purpura that is mostly located on the dependent parts like lower extremities and buttocks, arthralgia/arthritis, bowel angina, and hematuria/proteinuria. IgAV has been associated with a history of preceding infections, especially upper respiratory tract infection. IgAV is more common among children than adults (Davin 2011; Yang et al. 2014), but more severe in adults (Audemard-Verger et al. 2015). Histologically, IgAV is

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characterized by leukocytoclastic vasculitis associated with IgA immune complexes within affected organs, including the kidneys.

Patients with IgAN and IgAV with nephritis (IgAVN) have many of the same laboratory abnormalities and pathological features of renal biopsy specimens (Davin 2011; Jennette et al. 2013; Wyatt and Julian 2013; Yang et al. 2014; Audemard-Verger et al. 2015; Zhang et al. 2015). Thus, the pathogenesis of IgAN and IgAVN are considered to be similar. Since both diseases may be triggered by upper respiratory and/or gastrointestinal tract infections, mucosal immunity may play a key role in the development of the diseases (Davin 2011; Zhang et al. 2015). However, the reason for clinical differences between the two diseases is still unknown.

The toll-like receptor (TLR) family plays a critical role in the mammalian innate immune system, and is the first line of host defense against invading pathogens (Akira et al. 2006; Takeda and Akira 2015). In humans, this family now consists of 10 members (TLR1-TLR10) (Takeda and Akira 2015). Major microbial components binding to each TLR are as follows: bacterial triacyl lipopeptides for TLR1, bacterial peptidoglycan for TLR2, viral double-stranded RNA (dsRNA) for TLR3, bacterial lipopolysaccharide for TLR4, bacterial flagellin for TLR5, mycoplasma-derived diacyl lipopeptides for TLR6, viral single-stranded RNA (ssRNA) for TLR7 and TLR8, and bacterial and viral DNA for TLR9. The ligand for TLR10 currently remains unclear. Activation of TLR-mediated signaling pathways induces gene expression of inflammatory cytokines and Type I interferon (IFN-α/β) (Akira et al. 2006; Takeda and Akira 2015).

Abnormal expression levels of TLRs were shown in an animal model of IgAN (Suzuki et al. 2008) and in patients with IgAN and IgAV (Coppo et al. 2009; Chang et al. 2014; Donadio et al. 2014; Li et al. 2014). These studies investigated expression levels of TLRs in splenocytes or peripheral blood mononuclear cells (PBMCs) by real-time reverse transcription polymerase chain reaction (RT-PCR) and/or flow cytometry. Suzuki et al. (2008) investigated expression levels of *TLR2*, *TLR4*, and *TLR9* mRNAs in splenocytes from ddY mice, which develop IgAN spontaneously, and showed activation of the TLR9 pathway. Up-regulation of TLR4 was found in PBMCs from patients with IgAN by Coppo et al. (2009), children with IgAN and IgAV by Donadio et al. (2014), and children with IgAV by Chang et al. (2014). Li et al. (2014) reported up-regulation of TLR9 in PBMCs from IgAN patients. However, differences in expression patterns of major TLRs between IgAN and IgAVN are still unclear.

In the present study, patients with thin basement membrane nephropathy (TBMN) were enrolled as control patients without immune-mediated conditions. The common clinical manifestation of TBMN is persistent microscopic hematuria, without additional symptoms or progression to renal impairment. Light microscopy of renal samples shows almost normal glomerular histology, and direct immunofluorescent staining is negative for immunoglobulins and complements. Electron microscopy reveals thinning of the glomerular basement membrane (Tryggvason and Patrakka 2006).

In the present study, we examined expression levels of *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR9*, and *IFN-α* mRNAs in PBMCs from mainly adult patients with biopsy-proven IgAN and IgAVN by quantitative real-time RT-PCR in order to investigate the roles of TLRs in the pathogenesis of these two diseases. To our knowledge, this is the first study to show expression levels of *TLR5* mRNA in PBMCs from patients with IgAN and IgAVN.

Materials and Methods

Patients

We recruited 49 patients with IgAN, 20 patients with IgAVN, and 20 patients with TBMN as a control group, entering outpatient clinics of Akita University Hospital and its affiliated hospitals between 2007 and 2014. All patients were Japanese, and untreated prior to the start of the study. The clinical syndromes were based on the WHO recommendation (Churg et al. 1995). IgAN was diagnosed from renal biopsies on the basis of an increased prevalence of IgA deposits compared with those of other immunoglobulin classes (Tomino et al. 2003). All IgAN patients had no purpura or systemic signs of vasculitis or autoimmunity. A diagnosis of IgAVN was based on the presence of non-thrombocytopenic palpable purpura, associated with glomerulonephritis with IgA-dominant immune deposits (Audemard-Verger et al. 2015), confirmed by renal biopsy. TBMN was diagnosed by electron microscopy of renal biopsies, which showed diffuse thinning of the glomerular basement membrane width below 225 nm without depositions of immunoglobulins or complements within the glomeruli (Tryggvason and Patrakka 2006).

The protocol of this study was approved by the ethics committee of the institution involved (No. 1026), and informed consent for genetic studies was obtained from all subjects.

Laboratory assessment

Urinalysis data was assessed in each patient at the time of renal biopsy. We also assessed laboratory data, including serum levels of total protein, albumin, creatinine, IgA, and C-reactive protein (CRP). The estimated glomerular filtration rate (eGFR) was determined by a formula for Japanese patients (Matsuo et al. 2009).

Quantitative real-time RT-PCR

We quantified expression levels *of TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR9*, and *INF-α* mRNAs in PBMCs from each patient.

We collected blood samples in heparin tubes from patients at the time of renal biopsy. PBMCs were isolated by Ficoll-Conray density gradient centrifugation within several hours after blood sampling, and stored at −80°C until the start of analysis (for several months to several years). Total RNA was prepared with an RNeasy kit (Qiagen, Hilden, Germany) and used for cDNA synthesis with an oligo (dT) primer (Amesham Biosciences, Piscateway, NJ). PCR primers used in this study were previously described (Komatsuda et al. 2008) (Table 1).

Real-time RT-PCR reaction was performed in a final volume of 20 μl, containing 10 μl DNA Master Hybridization Probe 2× (Qiagen),

Table 1. PCR primers used in this study.

Primer name	Sequence	Tm (°C)
TLR2-FW	GGC CAG CAA ATT ACC TGT GTG	64
TLR2-RE	AGG CGG ACA TCC TGA ACC T	
TLR3-FW	CCT GGT TTG TTA ATT GGA TTA ACG A	62
TLR3-RE	TGA GGT GGA GTG TTG CAA AGG	
TLR4-FW	CTG CAA TGG ATC AAG GAC CA	62
TLR4-RE	TTA TCT GAA GGT GTT GCA CAT TCC	
TLR5-FW	TGC CTT GAA GCC TTC AGT TAT G	62
TLR5-RE	CCA ACC ACC ACC ATG ATG AG	
TLR7-FW	TTA CCT GGA TGG AAA CCA GCT ACT	62
TLR7-RE	TCA AGG CTG AGA AGC TGT AAG CTA	
TLR9-FW	TGA AGA CTT CAG GCC CAA CTG	62
TLR9-RE	TGC ACG GTC ACC AGG TTG T	
IFN-α-FW	TGC TTT ACT GAT GGT CCT GGT	60
IFN-α-RE	TCA TGT CTG TCC ATC AGA CAG	
GAPDH-FW	ATG GCT ATG ATG GAG GTC CAG	62
GAPDH-RE	TTG TCC TGC ATC TGC TTC AGC	

TLR, Toll-like receptor; FW, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; RE, reverse.

1 μl of 10 pmol forward and reverse primers, 1 μl of cDNA, and 7 μl of water, according to the manufacturer's instruction, as previously described (Komatsuda et al. 2008). After an initial denaturation step at 95°C for 900 sec, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 15 sec, hybridization at suitable temperatures for 20 sec, and elongation at 72°C for 20 sec, in a LightCycler (Roche Applied Science, Mannheim, Germany). A total 45 of cycles were performed. Each sample was run in triplicate.

For relative quantification of expression levels of *TLRs* and *INF-α* mRNAs, the expression level of *glyceraldehyde-3-phosphate dehydrogenase* mRNA was used as a control, as previously described (Komatsuda et al. 2008) (Table 1).

Statistical assessment

Clinical data were expressed as the mean ± standard deviation, or the median (range). The difference in the clinical data among diseases (IgAN, IgAVN, and TBMN) was analyzed by Pearson's chi-square test, Welch's *t* test, or Mann-Whitney's *U* test. The difference in expression levels of *TLRs* mRNAs among diseases was analyzed by Mann-Whitney's *U* test. The relationship between expression levels of *TLRs* mRNAs in IgAN patients and those in IgAVN patients was examined by the Spearman's correlation coefficient rank test. Univariate and multivariate logistic regression analyses were used to determine which *TLRs* mRNAs were associated with IgAN patients and which were associated with IgAVN patients. The Hosmer-Lemeshow test was used to evaluate the suitability of this analysis. The relationship between expression levels of *TLRs* mRNAs and laboratory parameters was examined by the Spearman's correlation coefficient rank test. All analyses were performed with SPSS version 11.0 software for Windows (Chicago, IL, USA). P values < 0.05 were regarded as significant.

Results

Clinical features in the studied patients

The characteristics of patients are shown in Table 2. The male/female ratio was significantly higher in IgAN patients than in IgAVN and TBMN patients. The mean ages were significantly higher in IgAN and IgAVN patients than in TBMN patients. Regarding laboratory parameters, there were significant differences among IgAN, IgAVN, and TBMN patients. Proteinuria levels, serum creatinine levels, and serum IgA levels were significantly higher in IgAN and IgAVN patients than those in TBMN patients, while eGFR levels were significantly lower in IgAN and IgAVN patients than in TBMN patients. Serum albumin levels were significantly lower in IgAN patients than in TBMN patients, and serum CRP levels were significantly higher in IgAVN patients than in IgAN and TBMN patients. Regarding the incidence of clinical syndromes, there were significant differences among IgAN, IgAVN, and TBMN patients. Persistent hematuria (with little or no proteinuria and no evidence of other features of nephritic syndrome) was more frequent in TBMN patients than in IgAN and IgAVN patients. Acute nephritic syndrome was more frequent in IgAVN patients than in IgAN patients, while chronic

Table 2. Summary of clinical characteristics of patients with IgAN, IgAVN, and TBMN.

	IgAN (n = 49)	IgAVN (n = 20)	TBMN (n = 20)	P-value†	P-value‡	P-value§
Male/female	27/22	5/15	5/15	0.023[a]	0.023[a]	1[a]
Age (years)	44.3 ± 17.7	51.1 ± 23.9	31.6 ± 13.9	0.261[b]	0.003[b]	0.004[b]
Proteinuria (g/gCr)	0.88 (0.06-8.01)	1.50 (0.05-7.90)	0.10 (0.00-1.85)	0.450[c]	< 0.001[c]	< 0.001[c]
Hematuria (/HPF)						
0-4	0	0	0			
5-9	8	2	2	0.498[a]	0.498[a]	1[a]
10-19	9	5	7	0.534[a]	0.137[a]	0.490[a]
20-49	15	4	4	0.371[a]	0.371[a]	1[a]
50-99	9	2	2	0.389[a]	0.389[a]	1[a]
>100	8	7	5	0.088[a]	0.403[a]	0.490[a]
Serum TP (g/dl)	6.9 (4.8-8.0)	7.1 (5.0-7.6)	6.9 (6.3-7.9)	0.730[c]	0.701[c]	0.841[c]
Serum Alb (g/dl)	4.0 (1.7-4.7)	4.0 (2.1-4.7)	4.3 (3.2-5.1)	0.889[c]	0.016[c]	0.050[c]
Serum Cr (mg/dl)	0.87 (0.5-2.5)	0.80 (0.5-1.2)	0.56 (0.4-0.8)	0.070[c]	< 0.001[c]	< 0.001[c]
eGFR (ml/min/1.73m^2)	73.1 ± 32.3	78.8 ± 31.6	115.7 ± 29.7	0.507[b]	< 0.001[b]	0.001[b]
Serum IgA (mg/dl)	335 (145-536)	371 (181-810)	160 (104-401)	0.519[c]	< 0.001[c]	< 0.001[c]
Serum CRP (mg/dl)	0.1 (0.0-8.2)	0.3 (0.0-5.2)	0.0 (0.0-3.6)	< 0.001[c]	0.114[c]	< 0.001[c]
Persistent hematuria	25	13	20	0.290[a]	< 0.001[a]	0.004[a]
Acute nephritic syndrome	0	2	0	0.025[a]		0.147[a]
Chronic nephritic syndrome	22	1	0	0.001[a]	< 0.001[a]	0.311[a]
Nephrotic syndrome	2	3	0	0.112[a]	0.359[a]	0.072[a]
RPGN	0	1	0	0.115[a]		0.311[a]

Continuous data are presented as the mean ± standard deviation, or median (range). Category data are presented as the number of patients.

Alb, albumin; Cr, creatinine; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HPF, high power field; IgAN, IgA nephropathy; IgAVN, IgA vasculitis with nephritis; RPGN, rapidly progressive glomerulonephritis; TBMN, thin basement membrane nephropathy; TP, total protein.

†Difference between IgAN and IgAVN; ‡Difference between IgAN and TBMN; §Difference between IgAVN and TBMN.

[a]Pearson's chi-square test; [b]Welch's *t* test; [c]Mann-Whitney's *U* test.

nephritic syndrome was more frequent in IgAN patients than in IgAVN and TBMN patients. Within 2 weeks before renal biopsy, most patients had no symptoms of infection, except for one IgAN patient with erysipelas and two IgAVN patients with upper respiratory tract infection.

Quantification of expression levels of TLR and IFN-α mRNAs in PBMCs from patients by real-time RT-PCR

We examined expression levels of *TLR* and *IFN-α* mRNAs in PBMCs from patients by real-time RT-PCR (Fig. 1). Relative expression levels of *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR9*, and *IFN-α* mRNAs were significantly higher in IgAN patients than those in TBMN patients. Relative expression levels of *TLR2*, *TLR3*, *TLR5*, *TLR7*, *TLR9*, and *IFN-α* mRNAs were significantly higher in IgAVN patients than in TBMN patients. Relative expression levels of *TLR4* mRNA were significantly higher in IgAN patients than in IgAVN patients. Finally, relative expression levels of *TLR5* and *TLR9* mRNAs were significantly higher in IgAVN patients than in IgAN patients.

To analyze which *TLR* mRNAs were associated with IgAN patients and which were associated with IgAVN patients, univariate and multivariate logistic regression analyses were performed (Table 3). In univariate logistic regression analysis, higher expression levels of *TLR4* mRNA (per 0.01 arbitrary units) and lower expression levels of *TLR9* mRNA were significantly correlated with IgAN patients. Multivariate analyses were performed on *TLR2*, *TLR3*, *TLR4*, *TLR5*, and *TLR7* mRNA, since there is an extremely strong relationship between *TLR5* and *TLR9* (Table 4). Higher expression levels of *TLR2* and *TLR3* mRNAs were significantly correlated with IgAN patients,

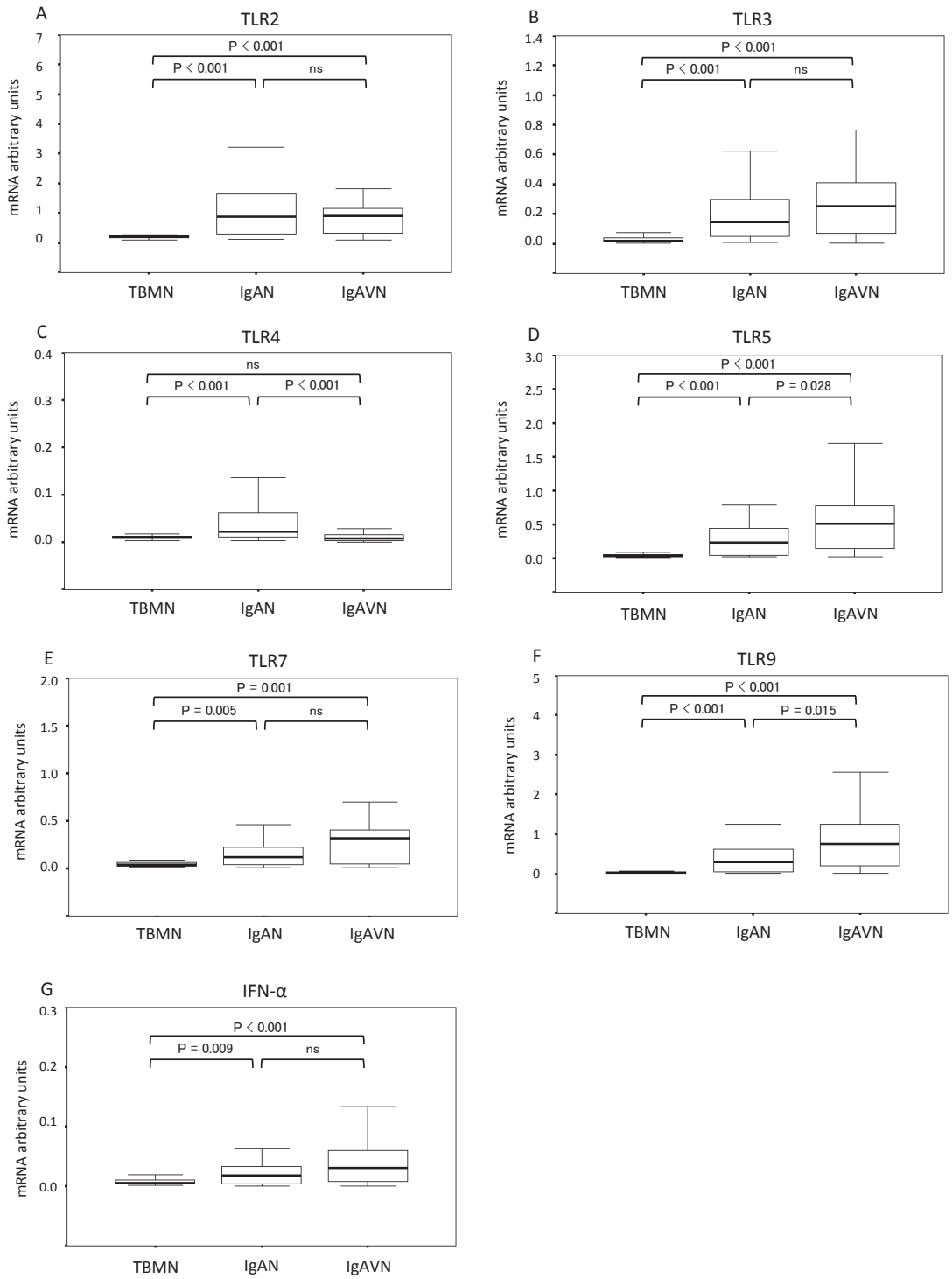

Fig. 1. Expression levels of *TLR* and *IFN-α* mRNAs in PBMCs from patients with IgAN, IgAVN, and TBMN. Box and whisker spots are shown: *TLR2* (A), *TLR3* (B), *TLR4* (C), *TLR5* (D), *TLR7* (E), *TLR9* (F), and *IFN-α* (G). The boxes represent the 25-75th percentiles, whereas the horizontal lines within each box represent the median values. The whiskers represent the lowest and highest values.

and lower expression levels of *TLR5* mRNA were significantly correlated with IgAN patients.

We compared patient's expression levels of *TLR* and *IFN-α* mRNAs in PBMCs with their laboratory parameters (Table 5). In IgAN patients, there were significant correlations between several laboratory parameters and expression levels of *TLR* mRNAs. Levels of proteinuria were correlated with expression levels of *TLR2*, *TLR3*, *TLR5*, and *TLR9* mRNAs. Levels of serum IgA were correlated with expression levels of *TLR4* mRNA. On the other hand, in

Table 3. Association of expression levels of *TLRs* mRNAs in IgAN and IgAVN patients.

	Univariate model (IgAN vs IgAVN)		Multivariate model†	
	OR (95% CI)	P-value	OR (95% CI)	P-value
TLR2	0.9 (0.9-1.0)	0.376	1.3 (1.0-1.7)	0.022
TLR3	0.9 (0.9-1.0)	0.440	2.0 (1.0-3.9)	0.042
TLR4	2.7 (1.4-6.2)	0.006	21.4 (0.8-555.5)	0.065
TLR5	0.9 (0.9-0.9)	0.054	0.3 (0.1-0.9)	0.030
TLR7	0.9 (0.9-1.0)	0.281	0.7 (0.6-1.0)	0.056
TLR9	0.9 (0.9-0.9)	0.041		

CI, confident interval; IgAN, IgA nephropathy; IgAVN, IgA vasculitis with nephritis; OR, Odds ratio; TLRs, Toll-like receptors.
†Multivariate logistic regression analyses of expression levels of *TLR2*, *TLR3*, *TLR4*, *TLR5*, and *TLR7* mRNAs.

Table 4. Correlations between expression levels of *TLRs* mRNAs in IgAN patients and those in IgAVN patients.

	TLR2	TLR3	TLR4	TLR5	TLR7	TLR9
TLR2	-	0.88**	0.46**	0.85**	0.81**	0.83**
TLR3		-	0.24*	0.95**	0.91**	0.95**
TLR4			-	0.19	0.20	0.14
TLR5				-	0.91**	0.98**
TLR7					-	0.92**
TLR9						-

IgAN, IgA nephropathy; IgAVN, IgA vasculitis with nephritis; TLRs, Toll like receptors. Only R_s values (correlation coefficients) are shown. *P < 0.05, **P < 0.0001.

IgAVN patients, there was no significant correlation between expression levels of *TLRs* and *IFN-α* mRNAs, and laboratory parameters (data not shown). Histological parameters, including glomerular crescents and sclerosis in IgAN and IgAVN patients, did not correlate with expression levels of any mRNAs (data not shown).

Discussion

Currently, information regarding signaling of TLRs in glomerular diseases is limited (Mao and Huang 2014). Tables 6 and 7 summarize previously reported data and our data of expression of TLR mRNAs in PBMCs from IgAN (Coppo et al. 2009; Donadio et al. 2014; Li et al. 2014) and from IgAV patients with or without renal involvement (Chang et al. 2014; Donadio et al. 2014). There are several differences in the backgrounds of enrolled patients among the previous studies and our study. In the studies of Donadio et al. (2014) and Chang et al. (2014), all patients were children, whereas in the studies of Coppo et al. (2009) and Li et al. (2014), and in our study, most patients were adults. In our study, and in previous studies (Coppo et al. 2009; Chang et al. 2014; Donadio et al. 2014), the diagnosis of IgAN was made by renal biopsy in all patients. Biopsies were also used to diagnose IgAVN in our study, but not in previous studies of IgAV in children (Chang et al. 2014; Donadio et al. 2014). In the studies of Chang et al. (2014) and Donadio et al. (2014), more than 50% of children did not show signs of nephritis.

In PBMCs from IgAN patients, we observed up-regulation of *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR9*, and *IFN-α* mRNAs. Similarly, up-regulation of *TLR4* mRNA and/or *TLR9* mRNA was also observed by Coppo et al. (2009), Donadio et al. (2014), and Li et al. (2014). Additionally, in our study, levels of proteinuria and serum IgA were correlated with expression levels of *TLR2*, *TLR3*, *TLR4*, *TLR5*, and *TLR9* mRNAs. Li et al. (2014) similarly found levels of serum IgA1 to be correlated with expression levels of *TLR9* mRNA.

In PBMCs from IgAVN patients, we observed up-regulation of *TLR2*, *TLR3*, *TLR5*, *TLR7*, *TLR9*, and *IFN-α* mRNAs. Similarly, Donadio et al. (2014) also observed up-regulation of *TLR2* mRNA. The differences in expression levels of *TLR3*, *TLR4*, and *TLR9* mRNAs between previous studies (Chang et al. 2014; Donadio et al. 2014) and our study may result in part from the different backgrounds of enrolled patients. In our study, expression levels of *TLR5* and *TLR9* mRNAs in PBMCs from IgAVN patients were significantly higher than in IgAN patients, while expression

Table 5. Correlations between clinical parameters and expression levels of *TLRs* mRNAs in IgAN patients.

	Median (range) or Mean ± SD		R	P-value
Proteinuria	0.88 (0.06-8.01) g/gCr	TLR2	0.288	0.045
		TLR3	0.297	0.039
		TLR4	0.131	n.s.
		TLR5	0.323	0.024
		TLR7	0.264	n.s.
		TLR9	0.303	0.034
		IFN-α	0.278	n.s.
Serum IgA	335 (145-536) mg/dl	TLR2	0.182	n.s.
		TLR3	0.088	n.s.
		TLR4	0.286	0.046
		TLR5	0.083	n.s.
		TLR7	0.142	n.s.
		TLR9	0.131	n.s.
		IFN-α	0.156	n.s.
eGFR	73.1 ± 32.3 ml/min/1.73m^2	TLR2	-0.227	n.s.
		TLR3	-0.137	n.s.
		TLR4	-0.173	n.s.
		TLR5	-0.159	n.s.
		TLR7	-0.042	n.s.
		TLR9	-0.138	n.s.
		IFN-α	-0.098	n.s.

Continuous data are presented as the median (range) or mean ± standard deviation.
Cr, creatinine; eGFR, estimated glomerular filtration rate; IgAN, IgA nephropathy; n.s., not significant; SD, standard deviation; TLR, Toll-like receptor.

Table 6. Summary of previous reports, and our findings, of expression levels of TLRs in IgAN patients.

Authors	Patients	n	Methods	TLR2	TLR3	TLR4	TLR5	TLR7	TLR9
Coppo et al. (2009)	Children & Adults	47	Flow cytometry/RT-PCR	ND	→	↑	ND	→	ND
Donadio et al. (2014)	Children	25	Real-time RT-PCR	→	→	↑	ND	ND	→
Li et al. (2014)	Adults	30	RT-PCR	ND	ND	ND	ND	ND	↑
Saito et al. (present study)	Adults	49	Real-time RT-PCR	↑	↑	↑	↑	↑	↑

IgAN, IgA nephropathy; ND, no data; RT-PCR, reverse transcription polymerase chain reaction; TLR, Toll-like receptor.

levels of *TLR4* mRNA in PBMCs from IgAVN patients were significantly lower than in those from IgAN patients.

As mentioned above, several previous studies, and our study, have demonstrated up-regulation of *TLs* mRNAs in PBMCs from IgAN patients and IgAV patients with or without renal involvement. Below, we discuss the possible roles of the activation of TLRs in the pathogenesis of these two related diseases.

TLR2 recognizes components from a variety of microbial pathogens, including peptidoglycan of Gram-positive bacteria and lipoproteins from Gram-negative bacteria (Akira et al. 2006; Takeda and Akira 2015). The mechanism by which TLR2 recognizes a wide variety of microbial components results from its cooperation with other TLRs (Takeda and Akira 2015). Lartigue et al. (2009) demonstrated critical roles of TRL2 and TLR4 in autoantibody

Table 7. Summary of previous reports, and our findings, of expression levels of TLRs in IgAVN patient.

Authors	Patients	n	Methods	TLR2	TLR3	TLR4	TLR5	TLR7	TLR9
Chang et al. (2014)	Children	105	Flow cytometry/	ND	→	↑	ND	ND	ND
Donadio et al. (2014)	Children	63	qPCR RT-PCR	↑	→	↑	ND	ND	→
Saito et al. (present study)	Adults	20	RT-PCR	↑	↑	→	↑	↑	↑

IgAVN, IgA vasculitis with nephritis; ND, no data; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; TLR, Toll-like receptor.

production and glomerulonephritis in a mouse lupus model. In our study, up-regulated expression levels of *TLR2* mRNA were observed in PBMCs from both IgAN and IgAVN patients. Furthermore, expression levels of *TLR2* mRNA were correlated with levels of proteinuria in IgAN patients. These findings suggest an important role of TLR2 in the pathogenesis of IgAN, as well as IgAVN.

TLR3 is implicated in the recognition of dsRNA, thereby detecting viral infection (Akira et al. 2006; Takeda and Akira 2015). Merkle et al. (2011) examined the effects of proinflammatory cytokines on the expression of TLR3 in cultured human mesangial cells infected with hepatitis C virus (HCV), and found TLR3-dependent regulation of cytokines in HCV-associated glomerulonephritis. In our study, up-regulated expression levels of *TLR3* mRNA were observed in PBMCs from both IgAN and IgAVN patients. Furthermore, expression levels of *TLR3* mRNA were correlated with levels of proteinuria in IgAN patients. These results suggest an important role of TLR3 in the pathogenesis of IgAN, as well as IgAVN.

TLR4 recognizes exogenous ligands, including lipopolysaccharide (LPS), from Gram-negative bacteria, and endogenous ligands, including heat shock proteins (HSP60 and HSP70), from damaged host cells (Akira et al. 2006; Takeda and Akira 2015). Brown et al. (2007) examined the effect of TLR4 ligands, a synthetic analogue of the active part of endotoxin and a highly purified LPS from *Escherichia coli* (*E. coli*), on the development of nephrotoxic antibody-mediated glomerulonephritis in a mouse model. TLR4 on both circulating leukocytes and glomerular mesangial cells were found to contribute to the inflammatory effects of antibody deposition within glomeruli. As TLR4 is specific for *E. coli* LPS, Coppo (2015) suggested a role for intestinal mucosa-associated lymphoid tissue as a promoter of systemic microinflammation in IgAN. In our study, up-regulated expression levels of *TLR4* mRNA were observed in PBMCs from IgAN patients, as similarly observed in previous studies (Coppo et al. 2009; Donadio et al. 2014). We also found that expression levels of *TLR4* mRNA were significantly correlated with levels of serum IgA in IgAN patients, but not in IgAVN patients. Furthermore, Shang et al. (2008) demonstrated that the number of IgA-producing plasma cells was increased in the lamina propria of transgenic mice expressing a constitutively active form of TLR4 in the intestinal epithelium. Lim et al. (2011) demonstrated that not only LPS-, but also IgA-treatment, up-regulated expression levels of *TLR4* mRNA in cultured mouse mesangial cells.

Conversely, expression levels of *TLR4* mRNA in PBMCs from IgAVN patients were significantly lower than in those from IgAN patients. This may relate to the different pathogenetic mechanisms between the two diseases. Canpinar et al. (2010) examined cell surface expression levels of TLR4 on PBMCs from patients with a clinical diagnosis of IgAV by flow cytometric analysis. Levels of TLR4 expression were significantly lower in IgAV patients compared with healthy controls when stimulated with LPS and HSP60. They suggested that lower TLR4 response to these ligands in IgAV patients may reflect a tolerance to bacterial antigens. The absence of up-regulated expression levels of *TLR4* mRNA in PBMCs from IgAVN patients in our study may be related to an underlying tolerance to exogenous and endogenous ligands during the course of disease.

TLR5 is responsible for the detection of flagellin, a monomeric constituent of bacterial flagella (Akira et al. 2006; Takeda and Akira 2015). There are several reports suggesting roles for flagellin/TLR5 responses in the pathogenesis of chronic inflammatory bowel disease and airway disease. Lodes et al. (2004) identified bacterial flagellin as a dominant antigen in patients with Crohn's disease and in murine colitis models. Yu et al. (2012) demonstrated that flagellin/TLR5 responses induce mucus hypersecretion in chronic inflammatory airway diseases. Our study found up-regulated expression levels of *TLR5* mRNA in PBMCs from patients with IgAN and IgAVN, and is to the best of our knowledge the first study to report this. It is possible that up-regulated expression levels of *TLR5* mRNA are involved in the pathogenesis of both IgAN and IgAVN. Although expression levels of *TLR5* mRNA in PBMCs were higher in IgAVN patients than in IgAN patients, they were correlated with levels of proteinuria in IgAN patients.

TLR7 recognizes ssRNA from viruses (Akira et al. 2006; Takeda and Akira 2015). Bacterial RNA from group B *Streptococcus* is also known to be recognized by TLR7 in the lysosomes of conventional dendritic cells (Mancuso et al. 2009). Pawar et al. (2006) demonstrated the effect of TLR7 ligation on exacerbation of immune complex glomerulonephritis in a mouse lupus model, and suggested a role for TLR7 in other types of infection-associated glomerulonephritis. In our study, up-regulated expression levels of *TLR7* mRNA were observed in PBMCs from both

IgAN and IgAVN patients. This finding suggests a role of TLR7 in the pathogenesis of IgAN and IgAVN.

TLR9 is essential for the recognition of the CpG motif of bacterial and viral DNA (Akira et al. 2006; Takeda and Akira 2015). As environmental pathogens are speculated to aggravate renal injury in IgAN, Suzuki et al. (2008) examined the relationship between expression levels of *TLR9* mRNA in splenocytes and disease activity of renal injury in IgAN in ddY mice. They revealed that the severity of glomerular injury in this model was correlated with expression levels of *TLR9* mRNA in splenocytes, and that nasal challenge with TLR9 ligands, CpG-oligodeoxynucleotides, aggravated renal injury and increased serum and mesangial IgA. These findings support the hypothesis that TLR9 may contribute the pathogenesis of IgAN. In our study, up-regulated expression levels of *TLR9* mRNA were observed in PBMCs from both IgAN and IgAVN patients. Furthermore, expression levels of *TLR9* mRNA were correlated with levels of proteinuria in IgAN patients. These findings suggest a role for TLR9 in the pathogenesis of IgAN, as well as IgAVN.

TLR-mediated signaling pathways induce gene expression of inflammatory cytokines and IFN-α/β (Akira et al. 2006; Takeda and Akira 2015). In our study, up-regulated expression levels of *IFN-α* mRNA were observed in PBMCs from IgAN and IgAVN patients. These findings support the hypothesis that IFN-α may trigger the pathogenic development of IgAN (Wardle 2004).

There are some limitations of the present study. We only checked expression levels of *TLR* and *INF-α* mRNAs, but did not analyze protein levels, cell surface expression, and TLR downstream signaling, except for IFN-α. Further study is required to elucidate the real contributions of these molecules in diseases.

In conclusion, we have revealed for the first time the different expression patterns of major *TLR* mRNAs in PBMCs from patients with IgAN and IgAVN. Our findings suggest that up-regulated expression levels of *TLR* mRNAs in PBMCs may play important roles in the development of IgAN and IgAVN, and that the different expression patterns between the two diseases may be related to their different pathogenetic mechanisms.

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Conflict of Interest

The authors declare no conflict of interest.

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